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13. ABSTRACT (Maximum 200 Words)
Ataxia Telangiectasia (A-T) is an autosomal recessive disease characterized by a progressive cerebellar ataxia, severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer. We are studying the biochemical function of ATM the product of the gene mutated in A-T in Xenopus, a simple model system suitable for cell cycle studies. We have cloned Xenopus ATM (X-ATM) and raised polyclonal antibodies against recombinant X-ATM that recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a Xenopus cell line. We found that X-ATM was expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes, and the protein and mRNA were present at relatively constant levels throughout development. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. The level of X-ATM protein did not change throughout the meiotic divisions or the synchronous mitotic cycles of cleavage stage embryos or following γ-irradiation of embryos. Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s).

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INTRODUCTION

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype (1, 2). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (3, 4, 5, 6, 7).

Cells from A-T patients show increased radiosensitivity to ionizing radiation (8), increased chromosomal loss and breakage, and abnormal telomere morphology (9, 10). Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle (11, 12, 13). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (14). ATM functions in the checkpoint pathway activated by DNA damage.

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use Xenopus as a novel model system to study both the biochemical role of ATM and its function during development. To enable such studies we have obtained a partial clone of the Xenopus homologue of the ATM protein (see 1998 annual summary).

The major objective of this proposal is to study the function of the ATM protein in a simple system in which biochemical analysis is a very powerful tool.

SUMMARY OF RESULTS

Polyclonal antibodies raised against recombinant X-ATM are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a Xenopus cell line (See 1998 annual summary).

We have purified these antibodies by affinity and used them to follow the temporal expression pattern of X-ATM protein during the highly synchronous meiotic and mitotic cell cycles of Xenopus laevis (Figure 1).We also have followed the expression of the protein during oogenesis and found that X-ATM was expressed maternally as early as stage II pre-vitellogenic oocytes (data not shown, see manuscript in press in the appendix). Subcellular fractionation showed that the protein was nuclear in both the female and male germlines (Figure 3). In addition, we did not observe any change in the level or mobility of X-ATM protein following γ–irradiation of embryos (data not shown, see manuscript in press in the appendix). Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s) (figure 3).

We have also designed better cell free systems to study the role of X-ATM in the S-phase cell cycle checkpoint. In this novel cell-free system genomic DNA is introduced in an extract along with naked DNA containing double strand breaks. The damaged DNA inhibits the replication of the genomic DNA in a dose-dependent manner, providing us with a rapid and very efficient way to follow the role of X-ATM in this process (data not shown). We are currently performing experiments that will help situate ATM within the cascade of events leading to cell cycle arrest following DNA damage.
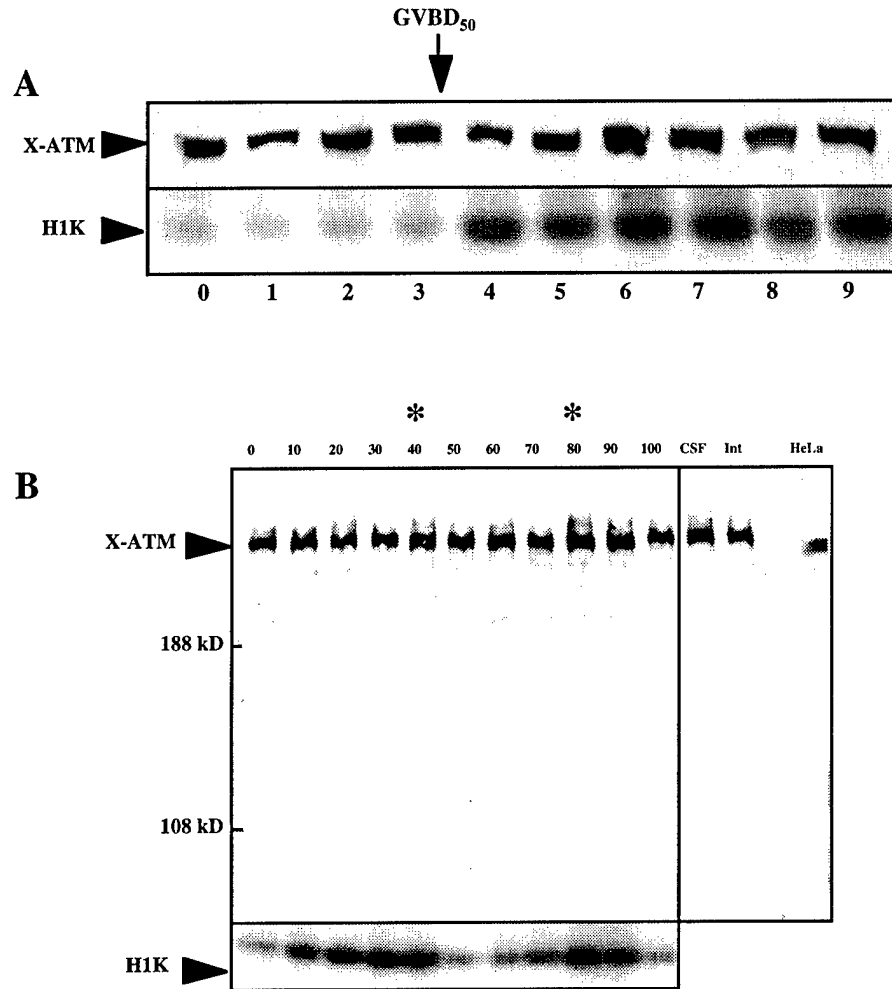
Figure 1: A: X-ATM expression throughout the meiotic cell cycle. Western blot analysis of oocytes progressing synchronously through meiosis was performed. t=0 is the time of addition of progesterone. $GVBD_{50}$ indicates the G2/M transition, the * indicates the interphase between metaphase of meiosis I and metaphase of meiosis II. X-ATM Western blot: top panel, Histone H1 kinase activity of the same samples : bottom panel.

B: X-ATM during the mitotic cell cyclesATM levels during S phase and mitosis were compared by western blot analysis of "cycling extracts" prepared from *Xenopus* eggs. X-ATM levels were monitored by 6% SDS-PAGE and western blot analysis of extracts over a period of 100 minutes (top panel), corresponding to two cell cycles as determined by analysis of cdc2/cyclin B kinase activity (bottom panel). M phase, i.e. the kinase activity peaks, is indicated by *. Comparison of a mitotic extract (CSF), and an interphase extract (INT), also indicated a similar amount of ATM in these different cell cycle phases. For comparison, a HeLa whole-cell lysate was also analyzed on this gel.
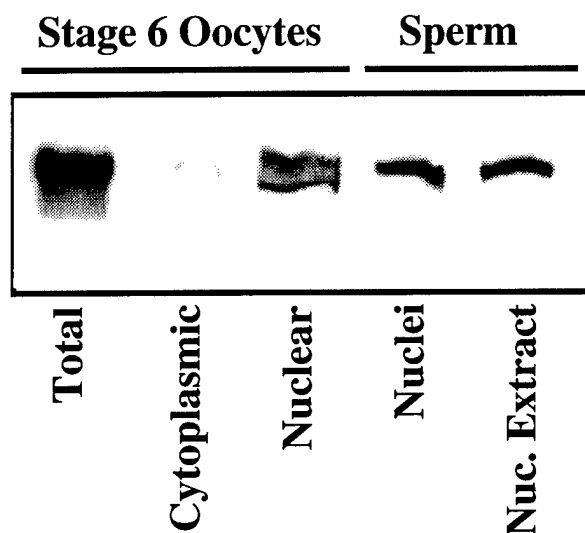
Stage 6 Oocytes   **Sperm**

Total   Cytoplasmic   Nuclear   Nuclei   Nuc. Extract

Figure 2: Subcellular localization of ATM in the germline. Protein samples from either complete stage VI oocytes (Total), stage VI oocyte cytoplasm (cytoplasm) or stage VI oocyte nuclei (nuclear) were processed for Western blotting. The two right lanes show a similar Western blot using either demembranated sperm nuclei or protein extracts prepared from nuclei.

Native   SDS + DTT

699 kD —

440 kD —   ◀ ATM-containing complex

◀ ATM monomer

232 kD —

140 kD —

Anti-X-ATM
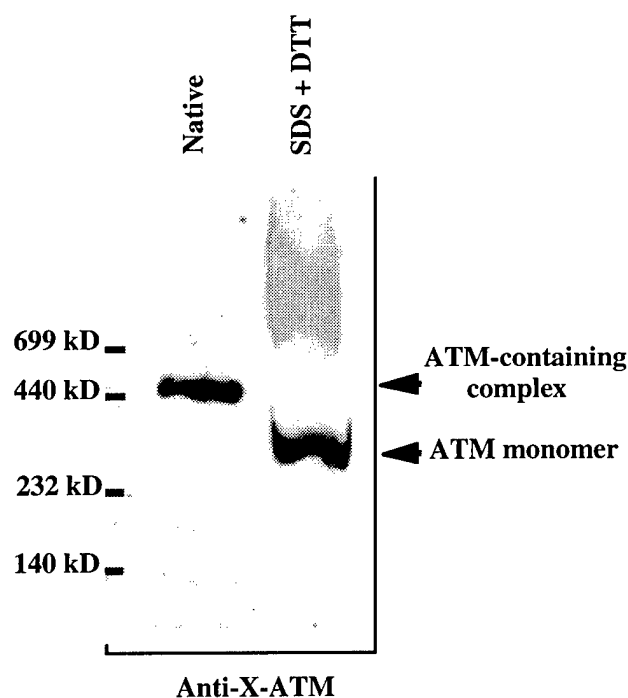
Figure 3: X-ATM complexes. 200 µg of egg extract were electrophoresed under native conditions and immunoblotted with X-ATM antibody (first lane). A band of approximately 450-500kD corresponding to the complex containing X-ATM is observed as indicated. The same sample was electrophoresed in the same polyacrylamide gradient gel under reducing and denaturing conditions (second lane). The immunoreactive band migrates at 370kDa, the molecular weight of monomeric ATM.

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CONCLUSIONS

We have continued our work to characterize the expression and the biochemical function of ATM in cell-free extracts derived from Xenopus eggs, in oocytes and in embryos. In addition we have started to develop cell-free assays to study the involvement of ATM in the S-phase cell cycle checkpoints. Finally we have established that ATM is present associated with other proteins in egg extracts. Part of our future work will be aimed at identifying these interacting proteins.

We have almost completed objective #1 of the SOW. This work has now been accepted for publication in "Oncogene" (see appendix). The experiments in which we proposed to look at the role of ATM in the G2 cell cycle checkpoint have given negative results. Since the proposal was written ATM has been strongly implicated in the S phase cell cycle checkpoint, therefore we have changed our strategy and developped a novel cell-free system to study this cell cycle checkpoint.

We have started objective #2 by demonstrating that ATM is associated with other proteins. This is a very important finding as it was the prerequisite that validates the feasibility of the objective. It was essential to establish this point before to start isolating the ATM interacting proteins.

Our studies using immunoprecipitation of ATM have not been successful. We have raised 2 different polyclonal antibodies against ATM which work very well on Western blot but are very poor at immunoprecipitating protein kinase activity from cell extracts. This has been a common problem working with ATM for different species.

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APPENDIX

Key Research Accomplishments:

- Cloning of the Xenopus homologue of human ATM.
- Expression of recombinant fragments of ATM for protein expression.
- Development and purification of polyclonal antibodies specific for Xenopus ATM.
- Description of the detailed temporal expression of ATM protein during the mitotic as well as meiotic cell cycles.
- Demonstration of the nuclear localization of ATM protein in the germlines.
- Demonstration that ATM is associated with other proteins.

Manuscripts and Abstracts:

Identification and Biochemical Characterization of a xenopus Homologue of ATM. **Jean Gautier**, Carmel Hensey and Kirsten Robertson. Eigth International Workshop on Ataxia-Telangiectasia. Las Vegas February 14-17, 1999.

Robertson K, Hensey C. and **Gautier J.** (1999). Isolation and Characterization of Xenopus ATM (X-ATM), Localization and Complex Formation During Oogenesis and Early Development. *Oncogene.* In Press.

Sequence accession number (Genbank):

The nucleotide sequence that was used to translate X-ATM has been deposited to GenBank, the accession number is: AF174488.

Isolation and Characterization of Xenopus ATM (X-ATM): Expression, Localization, and Complex Formation During Oogenesis and Early Development.

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ABSTRACT

ATM, the gene product mutated in Ataxia Telangiectasia (A-T) encodes a 350-kDa protein involved in the regulation of several cellular responses to DNA breaks. We used a degenerate PCR-based strategy to isolate a partial clone of X-ATM, the Xenopus homologue of human ATM. Sequence analysis and confirmed that the clone was most closely related to human ATM. Xenopus ATM protein (X-ATM) is 85% identical to human ATM within the kinase domain and 71% identical over the carboxyl-terminal half of the protein. Polyclonal antibodies raised against recombinant X-ATM are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a Xenopus cell line. We found that X-ATM was expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes, and the protein and mRNA were present at relatively constant levels throughout development. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. The level of X-ATM protein did not change throughout the meiotic divisions or the synchronous mitotic cycles of cleavage stage embryos. In addition, we did not observe any change in the level or mobility of X-ATM protein following γ–irradiation of embryos. Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s).

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INTRODUCTION

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype (Boder & Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (Athma et al., 1996; Chen et al., 1998; Stankovic et al., 1999; Swift et al., 1990; Yuille & Coignet, 1998).

Cells from A-T patients show increased radiosensitivity to ionizing radiation (Lavin & Shiloh, 1997), increased chromosomal loss and breakage, and abnormal telomere morphology (Smilenov et al., 1997; Vaziri et al., 1997). Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle (Beamish et al., 1996; Hoekstra, 1997; Meyn, 1995). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

The identification of a single mutated gene called ATM (Ataxia Telangiectasia Mutated) as the molecular basis for the phenotype has allowed a better understanding of both ATM function and the A-T pleiotropic phenotypes (Savitsky et al., 1995; Taylor, 1998). ATM is a nuclear phosphoprotein (Chen & Lee, 1996; Scott et al., 1998) and is a Serine/Threonine protein kinase for which the c-abl proto-oncogene, the replication protein RPA and the p53 tumor-suppressor gene have been identified as substrates in vitro (Banin et al., 1998; Baskaran et al., 1997; Canman et al., 1998; Gately et al., 1998; Khanna et al., 1998; Nakagawa et al., 1999; Shafman et al., 1997). The identification of these potential substrates have helped place ATM in a signal transduction pathway in which it could function in a cell cycle checkpoint through c-abl and p53.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (Hensey & Gautier, 1995). ATM functions in the checkpoint pathway activated by DNA damage. It has been shown that p53 is phosphorylated in an ATM-dependent fashion following DNA damage suggesting that p53 could be a direct substrate for the ATM protein kinase (Banin et al., 1998; Canman et al., 1998). Activation of p53 following DNA

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damage also involves dephosphorylation events that are dependent upon ATM activity (Waterman et al., 1998). In addition, studies in yeast and in mammalian cells also suggest that ATM might function as an upstream regulator of the Chk1 and Chk2 kinases (Brown et al., 1999; Chen et al., 1999; Matsuoka et al., 1998). However, the exact in vivo biochemical function of the ATM protein and its physiological substrates still remain elusive. In particular, the gap between our in vitro biochemical knowledge of the protein and its relationship with both cellular and patient phenotypes still awaits investigation.

A mouse model for ATM deficiency was created in several laboratories by specific germline inactivation of the ATM gene (Barlow et al., 1996; Elson et al., 1996; Herzog et al., 1998; Xu et al., 1996; Xu & Baltimore, 1996). Fibroblasts isolated from ATM$^{-/-}$ mice display similar cellular phenotypes to those observed in cells from A-T patients. As is the case with A-T patients, ATM deficient mice display a variety of growth defects, meiotic defects, immunological abnormalities, acute radiation sensitivity and cancer predisposition, confirming the pleiotropic roles of ATM.

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use Xenopus as a novel model system to study both the biochemical role of ATM and its function during development. The influence of DNA replication inhibition upon cell cycle progression (DNA replication or S-phase checkpoint) has been studied in detail in Xenopus egg extracts. First, it has been shown that threshold inhibition of DNA replication can elicit cell cycle arrest in egg extracts and prevent entry into mitosis (Dasso & Newport, 1990). Furthermore, both cdc25 and wee1 have been identified as modulators of this checkpoint. Upstream of cdc25, the protein kinase Chk1 and the 14-3-3 adapter protein have also been shown to be essential for cell cycle arrest (Kumagai et al., 1998a; Kumagai et al., 1998b).

To enable such studies we have obtained a partial clone of the Xenopus homologue of the ATM protein. We present its detailed expression patterns during the meiotic and the mitotic cell cycles as well as during development. We show that X-ATM is a maternal protein expressed as early as stage II oocytes and that ATM is exclusively nuclear in the germline. Finally, we present evidence that ATM exists in a large molecular weight complex in extracts derived from eggs.

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RESULTS

Isolation of a partial clone of the Xenopus homologue of ATM.

A protein alignment of the putative kinase domain of human ATM, S. pombe rad3, human ATR, S. cerevisiae TEL1, and D. melanogaster mei41 was used to identify regions of homology within the kinase domain of ATM and TEL1, that were not present in ATR, rad3 or mei41. Four amino acid stretches were identified and used to design degenerate oligonucleotides (see methods and Figure 1B). A 480 bp cDNA fragment was isolated by RT-PCR (Figure 1A) and sequencing of the fragment confirmed that it was most closely related to human ATM (Figure 1B). Using library screening, ATM-specific library construction, and 5'RACE (Figure 1A) additional sequence corresponding to approximately 50% of the predicted Xenopus ATM ORF was obtained.

An alignment of the carboxyl-terminal half of X-ATM with human ATM is shown in figure 1B. ATM is highly conserved between Xenopus and human with an overall identity of 71% in the region we have sequenced. Within the kinase domain the identity is 85% while the region upstream of the kinase domain (amino acids 1 to 1100 of X-ATM) shows 66% identity to the human protein. In contrast, when this region from X-ATM (a.a. 1 to 1100) is aligned with S.c. TEL1, S.p. rad3, D.m. mei41 or H.s. ATR, only short stretches of weak homology (ranging from 20 to 24%) are detected.

Characterization of X-ATM antibodies

Using an E.coli expression vector, we produced a 20-kDa fragment of the X-ATM cDNA corresponding to the region immediately 5' of the protein kinase domain (PQE60/TBH6H of Figure 1A). We chose this region as it had been previously shown to elicit a good immune response for human ATM (Brown et al., 1997). Additionally, we wanted to avoid raising antibodies against the conserved kinase domain which could potentially cross-react with other members of the ATM/PI3 kinase family. This 20-kDa polypeptide was expressed in E. coli and was used as an antigen for antibody production in rabbits (See Materials and Methods).

Programmed reticulocyte lysate containing the 65-kDa carboxyl terminal portion of X-ATM (T7/TBH-XTC of Figure 1A) was used to screen the different antisera. Sera from both rabbits

showed strong cross-reactivity with the translated product (data not shown) and recognized a protein of approximately 370-kDa in Xenopus egg extracts (Figure 2A, lanes 1 and 2). Due to the presence of a 210-kDa cross-reacting polypeptide in serum A (Figure 2A, lane 1), we used serum B for the remaining experiments. Preincubation of the antiserum with the 20-kDa polypeptide used for immunization eliminated the signal, confirming the identity of the protein recognized by the antiserum (data not shown). Moreover, we were able to immunoaffinity purify the X-ATM antibody from rabbit B using the 20-kDa polypeptide coupled to agarose. This purified antibody, which was used in all further experiments, detected a single polypeptide in Xenopus egg extracts that migrated slightly slower than human ATM (compare lanes 3 and 4, figure 2A). It also immunoprecipitated a fraction of the X-ATM protein from extracts as shown in figure 2B (lane 2).

Expression of X-ATM during early development.

Little is known about ATM expression during the early phases of vertebrate development. We therefore examined the temporal expression of both X-ATM mRNA and protein between fertilization and the swimming tadpole stage in Xenopus. Figure 3A shows an RNAse protection assay for the XTC cell line (Pudney *et al.*, 1973) and the indicated stages of development. X-ATM mRNA was detected in unfertilized eggs and expressed at relatively constant levels throughout cleavage, gastrulation, neurulation and the tadpole stage, although we consistently detected higher X-ATM mRNA levels in unfertilized eggs. Since transcription of zygotic genes does not start until the midblastula transition (St. 8-9), this demonstrates that X-ATM mRNA is maternally inherited. The temporal expression of X-ATM protein during similar developmental stages is shown in figure 3B. The protein is expressed maternally and throughout development where we observe a single cross-reacting polypeptide at all stages. Some variability in the X-ATM protein amounts was detected, particularly during neurulation. This might reflect developmentally regulated changes in the amount of X-ATM. Alternatively, this could also be due to the subcellular localization of the protein leading to different yields following embryonic extract preparation. We estimated the concentration of X-ATM to be 200 nM in egg extracts and 40 nM in unfertilized eggs (see Materials and Methods).

Subcellular localization of the ATM protein in the germline.

X-ATM is present in egg extracts and in unfertilized eggs (Figure 2A and 3B). We examined whether the protein was already present in the oocyte as opposed to being synthesized during meiotic maturation as has been described for some proteins (Sagata *et al.*, 1988). Xenopus oocytes were manually dissected from pieces of ovary and protein extracts were made at the stages of oogenesis indicated (Dumont, 1972). X-ATM is already present in previtellogenic oocytes (stage II) and persists throughout oogenesis (Figure 4A). Although the concentration of X-ATM per mass of tissue does not vary significantly throughout oogenesis, the mass per oocyte increases dramatically since the volume of the oocytes increases by a factor of 10^3 between stages I-II and stage VI.

Stage VI Xenopus oocytes contain a large (200 μm diameter) nucleus, known as the germinal vesicle (GV), which can be used to store nuclear proteins important for later development (Dreyer & Hausen, 1983). The GV can be isolated surgically from its surrounding cytoplasm, allowing the preparation of pure cytoplasmic and nuclear fractions from oocytes (Ford & Gurdon, 1977). We demonstrate that X-ATM is entirely nuclear in stage VI oocytes (Figure 4B). Moreover, using either demembranated sperm nuclei, or a nuclear extract prepared from these nuclei following their incubation in extracts (Walter *et al.*, 1998), we show that X-ATM is also nuclear in the male germline.

Expression of X-ATM protein throughout the meiotic and mitotic cell cycles.

In Xenopus oocytes the highly synchronous meiotic cell cycle is easily studied in vitro, where progesterone induced oocyte maturation culminates in the completion of meiosis I and the progression to metaphase of meiosis II. To determine whether X-ATM protein levels fluctuated during the meiotic cell cycle, we performed Western blots on protein samples from oocytes synchronously undergoing meiosis. X-ATM protein levels remained constant throughout meiosis (Figure 5A). In this experiment, prophase-arrested oocytes were induced to undergo meiotic maturation in vitro by progesterone. Nuclear envelope breakdown took place at 3.5 hrs, meiosis I occurred at 6 hrs and the oocytes were arrested in metaphase of meiosis II by 9 hrs.

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Next, we followed X-ATM levels throughout the mitotic cell cycle which oscillations were assessed by following cdc2/cyclinB activity. The naturally synchronous cell divisions of the early embryo allow the preparation of cell free extracts that undergo cell cycle oscillations in vitro. Using such extracts we examined X-ATM protein levels throughout these highly synchronized cell cycles. ATM is expressed throughout the cell cycle and its levels remain constant over the course of two cell cycles (Figure 5B). Additionally, there was no change in the electrophoretic mobility of X-ATM.

X-ATM protein in γ-irradiated eggs

In Xenopus, irradiation of cleavage stage embryos leads to massive and synchronous apoptosis at the onset of gastrulation (St. 10.5) (Anderson *et al.*, 1997; Hensey & Gautier, 1997). To assess whether this response correlated with changes in the amounts or the electrophoretic mobility of X-ATM, we compare the levels of X-ATM in protein samples from untreated and γ-irradiated embryos between stage 8 and 10.5. X-ATM protein levels were similar in control and irradiated embryos. In addition no change in the mobility of the protein was detected (Figure 6).

X-ATM is present in a protein complex in Xenopus extracts.

The carboxyl-terminal kinase domain of ATM represents a small portion of this large protein. Little is known about the function of the domain(s) outside the kinase region but support for its importance comes from both genetic analysis of ATM mutants (Concannon & Gatti, 1997) as well as the finding that a putative leucine zipper region of ATM behaved as a transdominant negative (Morgan *et al.*, 1997). A central proline rich domain has already been shown to interact with the c-abl SH3 domain (Shafman *et al.*, 1997), and the remainder of the protein is suspected of playing regulatory functions by interacting with other proteins. To determine whether X-ATM might be associated with other proteins in Xenopus extracts we analyzed extracts by native gel electrophoresis. We detected a native, high molecular weight X-ATM-containing complex with an estimated size of 500-kDa (Figure 7). In addition, using sucrose gradient centrifugation, we detected a high molecular weight form of ATM in extracts consistent with our electrophoresis analysis (data not shown).

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DISCUSSION

Xenopus ATM

We report the cloning of a Xenopus homologue of ATM, the gene mutated in Ataxia-Telangiectasia. Although we have not yet isolated the full-length clone, sequence comparison spanning 50% of the predicted ORF, establishes clearly that we have isolated a Xenopus ATM homologue rather than a member of the PI3-kinase family. The amino acid levels of identity between X-ATM and human or mouse ATM are 71% and 69%, respectively while human ATM is 85% identical to mouse ATM over the same half of the protein. In comparison, there is only weak homology outside the kinase domain between X-ATM and ATR, mei41 or rad3, three genes which are forming the "ATR/rad3" branch of this family of protein kinases (Hoekstra, 1997).

. The high degree of conservation between X-ATM and ATM within the kinase domains (85% identity) strongly suggests that the catalytic activity of these two proteins will be conserved. The decreasing levels of identity outside the catalytic domains could reflect potential differences in regulation of these proteins between species.

Mutations in the ATM gene are not clustered in a specific area of the molecule or restricted to a limited numbers of residues, instead they span the entire ORF of the molecule. This has made it difficult to discriminate between true mutations and polymorphisms. The availability of X-ATM sequence might help resolve some of these issues by identifying mutations in conserved residues that have been originally classified as polymorphisms. Conversely, it might help confirm the location of polymorphisms in non-conserved residues.

Cell cycle patterns of X-ATM expression

We present a detailed analysis of the expression of X-ATM throughout both meiotic and mitotic cell cycles. These cell cycles are naturally synchronous in Xenopus and allowed the cell cycle expression of ATM to be followed in undisturbed cell cycles. We find no evidence for cell cycle oscillation in the level or the electrophoretic mobility of ATM protein during the mitotic cell cycles in cell-free extracts or during the meiotic divisions of the oocyte. This confirms previous results for human ATM obtained in cells that have been synchronized in culture (Brown *et al.*,

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1997; Gately *et al.*, 1998). Due to the large size of X-ATM, small variations in mobility might not be detectable despite the use of very resolutive PAGE. In addition, ATM could be modified in ways that does not affect its electrophoretic mobility. Therefore we cannot rule out that X-ATM protein could be modified in a cell cycle dependent manner.

Developmental expression of X-ATM

We found that X-ATM was expressed maternally, very early during oogenesis. This might reflect the need for ATM function in growing oocytes and during meiosis. Later during embryonic development we did not observe dramatic changes in the levels of mRNA or protein which is consistent with X-ATM playing a role in all cell types throughout development. RNAse protection assays show the presence of a second protected fragment at stages 9 and 10 of smaller size than the expected protected fragment. This could be due to the 2 different mRNA species generated by alternative splicing. Alternatively, a stable secondary structure could exist at either end of the protected fragment giving rise to the observed pattern. Since alternative mRNA splicing has not been observed within the ORF of human ATM (Savitsky *et al.*, 1995) we favor the second hypothesis.

We also followed the protein expression patterns of ATM in embryos that have been irradiated by ionizing radiation. We previously reported that such an insult induces a synchronous apoptotic program in Xenopus embryos, manifest at the onset of gastrulation (Anderson *et al.*, 1997; Hensey & Gautier, 1997). In such embryos destined to die by apoptosis we did not observe any changes in the levels of X-ATM. Moreover, when we followed X-ATM in Xenopus apoptotic extracts containing active caspase (as seen by PARP cleavage), we did not observe X-ATM cleavage despite the presence of a good consensus site for caspase cleavage (towards the C-terminal-end) of the X-ATM molecule (DIVD) (data not shown).

Localization

We clearly demonstrated that X-ATM is exclusively nuclear in the germline. Xenopus oocyte nuclei can be manually isolated from their surrounding cytoplasm with virtually no cross contamination. This provides unequivocal evidence for the subcellular localization of X-ATM in this system. Although X-ATM isolated from oocytes migrated with the same mobility on PAGE as

X-ATM isolated from eggs (see figure), we occasionally observed a second slower migrating band on Western blots from oocytes early during vitellogenesis (data not shown).

As meiotic maturation takes place and the nuclear envelope breaks down, X-ATM becomes cytoplasmic. In the unfertilized egg, corresponding to the second meiotic metaphase, the protein is cytoplasmic as seen by its presence in cell-free extracts. The nuclear localization of the protein in oocytes might reflect the fact that X-ATM could be essential for the proper completion of meiosis as is the case in mammals (Barlow et al., 1998; Xu et al., 1996). However, during the early cleavages of the embryos, the rapid divisions lack cell cycle checkpoints and are relatively insensitive to either DNA replication inhibitors or DNA damage (Hensey & Gautier, 1997). It will be interesting to determine whether exclusion of ATM from the nucleus correlates with the lack of cell cycle checkpoints and at what time during development X-ATM becomes nuclear again.

. It is interesting to compare the expression and localization of X-ATM with that of Xenopus p53 since in mammalian systems p53 has been shown to be a target and a substrate for the ATM protein kinase. Xenopus p53 is structurally and functionally related to human p53 (Cox et al., 1994; Soussi et al., 1987; Wang et al., 1995). Like X-ATM, p53 is present in oocytes and synthesized during early oogenesis (Tchang et al., 1993). However in contrast to what we observe for X-ATM, Xenopus p53 is entirely cytoplasmic in the oocyte. The difference in sub-cellular localization between p53 and X-ATM might provide a partial explanation for the unusually high levels of p53 protein observed in Xenopus oocytes and embryos.

Complex formation

We also clearly demonstrate that ATM is present in at least one larger complex than the monomeric form. The non-denaturing gels we used in these experiments allowed us to clearly resolve a complex of approximately 500 kDa, which could be disrupted following SDS treatment. It is possible that other larger complexes also exist but cannot be resolved using PAGE. We confirmed these findings using sucrose gradient centrifugation of egg extracts followed by Western blotting. We found that X-ATM was found in a high molecular weight complex sedimenting around 600 kDa (data not shown).

11

The finding that X-ATM is associated with other partner(s) in the simple, unregulated cell cycles of Xenopus cell-free extracts suggest that X-ATM might always require the association with regulatory subunits to perform its functions. ATM has been shown to interact with c-abl which is also a substrate for the kinase. It was also demonstrated that ATM interacts with β-adaptin, a protein involved in clathrin-mediated endocytosis of receptors (Lim *et al.*, 1998). The identity of the Xenopus ATM partner(s) awaits further investigation.

MATERIALS AND METHODS

Cloning of Xenopus *Atm*

Degenerate RT-PCR: Four Degenerate primers were designed from regions of amino acid identity between the kinase domain of ATM and its yeast homologue TEL 1, with a slight bias towards the human sequence. Primers 1 and 2 were in the 5'-3' direction. Primers 3 and 4 were in the 3'-5' direction. The primer sequences were as follows:

Primer 1: 5'-GCGCGGATCCGA(CT)GA(CT)(CT)T(AGCT)(AC)G(AGCT)CA(AG)GA(CT)-3' (amino acids DDLRQDA, positions 2719 to 2726 in ATM)

Primer 2: 5'-GCGCGGATCCT(AGCT)ATG(GC)A(AG)CA(AG)GT(AGCT)TT(CT)-3' (amino acids MQQVFQ, positions 2727 to 2733 in ATM)

Primer 3: 5'-GCGCATCGATA(CT)(AGCT)CC(AGCT)A(AG)(AG)TC(ATG)AT(AG)TG-3' (amino acids HIDLGV, positions 2886 to 2892 in ATM)

Primer 4: 5'-GCGCATCGATTT(AGCT)CC(CT)TG(AGCT)TC(AG)AA(AGCT)GC-3' (amino acids AFEQGK, positions 2892 to 2898 in ATM)

Total RNA was isolated from Xenopus Tissue Culture (XTC) cells (RNAzol B, Tel-Test) and was subjected to a reverse transcription reaction. The cDNA product was used as a template in PCR with primers 1 and 4 in a standard PCR reaction. A fragment of approximately 480 bp was amplified, and reamplified with nested degenerate primers 2 and 4.

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Library Screens: A λ Zap library from XTC cells was screened using the 480 bp PCR fragment as a probe and a high stringency hybridization was performed. Following 3 successive rounds of screening the clone XTC 3 was isolated containing a 2.1 kb insert including 600 bp of 3' UTR.

A further 500 bp of *X-Atm* was cloned upon screening of a second λ Zap library from Stage 25 Xenopus embryos (A gift from Dr. Hemmati-Brivanlou and Dr. Harland). The TBH 4 clone was 1 kb in size and partially overlaps with the 5' 500 bp of clone XTC 3 (see Figure 1A)

X-Atm Library construction: A Zap Express library was constructed using the Zap Express cDNA Gigapack III Gold Cloning kit, (Stratagene). Messenger RNA was isolated from XTC cell total RNA, and subjected to first strand synthesis using a primer specific for *X-Atm* (instead of the oligo DT primer provided) containing a XhoI site 5'-GAGAGAGAGACTAGTCTCGAGTGTGTCGACAATGTGACGTGC-3'. All cDNA's with molecular weights above 1 kb were excised, packaged into λ arms and the library titrated.

Subsequent screening of 20,000 plaques revealed a 2.5-kb clone, *X-Atm1.3* that overlaps with the 5' 210 bp of the TBH 4 clone (Figure 1A).

5' RACE: mRNAs were isolated from XTC cells as previously mentioned and reverse transcribed. Second strand synthesis and adapter ligation was carried out using the Marathon 5' RACE kit (Clontech). Long distance PCR was performed on the cDNA. After 3 rounds of PCR with nested primers an 800 bp product, clone RACE 1, was generated and resulted in a further 500 bp of new sequence (350 bp of this clone overlaps with the 5' of clone *X-Atm1.3*, see Figure 1A).

The assembly of all preceding clones provided 5.3 Kb of sequence corresponding to the 3'UTR and 50% of the predicted ORF of the cDNA. Amino acid sequence alignments and alignment scores were performed using Geneworks software.

RNAse Protection Assay

The RNAse protection assay was performed according to the protocol described in the RPAII kit (Ambion). A 480-bp fragment from clone TBH 4 (nucleotide position 5 to 485) was used as a probe (Figure 1A). The probe was hybridized with 15 μg of XTC cell total RNA and with RNA from samples of 10 Xenopus eggs or embryos, stages 5, 9, 10, 17 and 26. After hybridization the samples were digested with RNAse A and H, and analyzed by PAGE.

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Bacterial Expression of X-ATM Fragment and Generation of X-ATM Antibodies

A 500 bp fragment from X*Atm* clone TBH 4 (positions 5 to 485) was amplified by the forward primer 5'-GACGTCCTTAATAATCTGATT-3' upstream of an internal Nco I site and the reverse primer 5'-GCGCGGATCCGAGATTAAGGCCCCCGGCCAG-3' which contains a Bam HI site (Figure 1A) and cloned into the Nco I and Bam HI sites of the His_6 Expression vector PQE60.

We also cloned the 3' 1.5kb of the *X-Atm* ORF into a T7 vector that allowed the production of a 65-kDa polypeptide in rabbit reticulocyte lysate (T7/TBH-XTC).

X-ATM was expressed in *E. coli* and affinity purification of the denatured peptide was carried out as described in the QIAexpress protocol (Qiagen). 2 mg of protein was run on a 15% preparative polyacrylamide gel, the band excised from the gel and used to immunize rabbits.

Sera were tested by western blotting against the in vitro translated X-ATM. Sera from two rabbits gave a strong signal and the antibodies form rabbit B were further purified on an affinity column prepared by coupling the 20 kDa X-ATM protein fragment to CNBr sepharose beads.

Protein Sample Preparation

Cycling and CSF extract: Cycling extracts were prepared from unfertilized Xenopus eggs, as described in (Murray, 1991).

Embryos: Batches of 20 embryos from the indicated stages were collected and snap frozen in liquid nitrogen. The embryos were crushed in a modified RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.05% SDS) containing pepstatin, leupeptin, aprotinin and AEBSF (10μg/ml each). 100 μg of protein from each sample used in western blots.

Oocytes: Stages II, IV, and VI (Dumont, 1972) were gently crushed in 150 mM NaCl, 50 mM Tris, pH 7.4 and processed in the same way as embryos. Oocyte maturation, i.e. meiosis, was induced in vitro by 10 μM progesterone in MBS-H (Gautier & Maller, 1991).

Cells: XTC cells were washed in ice-cold PBS, pelleted and resuspended in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Nuclear Protein Extracts (NPE) were prepared according to (Walter *et al.*, 1998).

Western Blotting

Protein samples were separated under denaturing conditions on 20 cm, high resolution 6% SDS polyacrylamide gels, and transferred to nitrocellulose. After blocking in 5% milk in PBS, membranes were incubated with either affinity purified X-ATM antibody or human ATM antibody AT 1.8 (a gift from Dr. T. Yen), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Amersham).

Quantitation of X-ATM in Xenopus embryos

Linear T7/TBH-XTC clone (Figure 1A) was used as a template in an in vitro transcription/translation reaction with 2 µl of ^{35}S methionine of known specific activity. 1 µl samples from the completed reaction were counted in a scintillation counter to determine the average number of counts corresponding to the calculated number of moles of ^{35}S-Met. The amount of ^{35}S-Met incorporated into the X-ATM band was then determined by electrophoresing 1-3 µl of the translation reaction on a 10 % SDS-PAGE gel and transferring to nitrocellulose. The radiolabelled band was excised and counted, and counts were converted to pmoles of ^{35}S per µl of the translation. Western blots of both the translation products and CSF extract were carried out in parallel and the band intensities of different dilutions were compared and hence, knowing the number of methionines in the translation product, we were able to determine the molarity of X-ATM in CSF extract. This was extrapolated to embryos by comparing band intensities in western blots containing CSF and extracts from a known number of embryos resulting in an estimation of the Molar concentration of X-ATM per embryo

Native gradient gel electrophoresis

10 µl of interphase extract were either suspended in TBE containing 10 % glycerol or a modified buffer also containing 3 % SDS and 100 mM DTT. Samples in the SDS/DTT containing buffer were heated to 80°C for 5 minutes. Proteins were then separated on a non-denaturing linear gradient polyacrylamide gel in 1X TBE overnight. Before transferring proteins to nitrocellulose the gel was soaked in SDS buffer (48 mM Tris, 39 mM glycine, 0.25 % SDS) for 30 minutes at room

temperature with agitation, followed by a 30 minute soak at 80°C (Hendrickson *et al.*, 1996). Proteins were probed with the X-ATM antibody as previously described.

FIGURE LEGENDS

Figure 1.

A: ATM domains and cloning strategy.

The human ATM protein is represented by a thick black line, specific domains are indicated. X-ATM clones are indicated by arrows. T7/TBH-XTC denotes the in vitro translated fragment of X-ATM used to screen sera from rabbits A and B. PQE60/TBH6H was used in antigen production and part of this region was used in RNAse protection assays (construct T7/TBH-XTC)

B: Sequence comparison of X-ATM and human ATM.

50% of the predicted sequence of Xenopus ATM (X-ATM) was aligned with the amino acid sequence of human ATM (H-ATM) using Geneworks software (Intellegenetics). Identical amino acids between the 2 proteins are indicated in bold letters below the comparison. The regions of the human sequence that were used to derive degenerate oligonucleotides are underlined (thin lines). The PI3 kinase domain consensus region is underlined (thick line) . The nucleotide sequence that was used to translate X-ATM has been deposited to GenBank, the accession number is: AF174488.

Figure 2. Characterization and specificity of antibodies against X-ATM.

A: Specificity of the crude serum and the affinity purified antibody for X-ATM.

100 μg of Xenopus egg extract was electrophoresed (6 % SDS-PAGE) and Western blotted with the crude serum A (lane 1), the crude serum B (lane 2) or the affinity purified antibody from serum B (lane 3). In all cases a polypeptide of 370kDa was detected. Lane 4 is a human cell extract (HeLa) probed with an anti-human ATM antibody (Gately *et al.*, 1998).

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B: Immunoprecipitation of native X-ATM protein.

500 µg of extract were subjected to immunoprecipitation followed by Western blotting using the X-ATM antibody (lane 2). A 370 kDa polypeptide of the same size as that in the control extract (lane 1) is detected.

Figure 3.

A: Expression of X-ATM mRNA during development.

RNAse protection assays using a 480bp probe from clone TBH4 of the X-ATM cDNA were performed using standard protocols at different stages of development (as indicated above the lanes) and from RNA prepared from the Xenopus XTC cell line (1st lane). The protected band is 480bp.

B: Expression of X-ATM protein during Development.

. Equal amounts of protein extracts prepared from different stages were separated by electrophoresis followed by Western blot with the ATM affinity purified antibody. The protein is detected from stage 1 and the level of expression increases slightly through gastrulation (St. 13), and peaks at the time of formation of the neural tube and somites (St. 15-25), to slowly decrease thereafter. Drawings of the embryonic stages are shown above the autoradiogram.

Figure 4.

A: X-ATM during oogenesis.

Manually dissected oocytes at different stages of vitellogenesis were collected. Equal amounts of protein were separated by electrophoresis and subjected to Western blotting. The stages of oogenesis are indicated below the lanes. The mobility was compared with that of X-ATM in a CSF extract (CSF).

B: Subcellular localization of ATM in the germline.

Protein samples from either complete stage VI oocytes (Total), stage VI oocyte cytoplasm (cytoplasm) or stage VI oocyte nuclei (nuclear) were processed for Western blotting. The two right lanes show a similar Western blot using either demembranated sperm nuclei or protein extracts prepared from nuclei.

Figure 5.

A: X-ATM expression throughout the meiotic cell cycle.

Western blot analysis of oocytes progressing synchronously through meiosis was performed. t=0 is the time of addition of progesterone. $GVBD_{50}$ indicates the G2/M transition, the * indicates the interphase between metaphase of meiosis I and metaphase of meiosis II. X-ATM Western blot: top panel, Histone H1 kinase activity of the same samples : bottom panel.

B: X-ATM during the mitotic cell cycles

ATM levels during S phase and mitosis were compared by western blot analysis of "cycling extracts" prepared from *Xenopus* eggs. X-ATM levels were monitored by 6% SDS-PAGE and western blot analysis of extracts over a period of 100 minutes (top panel), corresponding to two cell cycles as determined by analysis of cdc2/cyclin B kinase activity (bottom panel). M phase, i.e. the kinase activity peaks, is indicated by *. Comparison of a mitotic extract (CSF), and an interphase extract (INT), also indicated a similar amount of ATM in these different cell cycle phases. For comparison, a HeLa whole-cell lysate was also analyzed on this gel. Human ATM antibody (Gately, D.P., Hittle J.C., Chan, G.K.T., and Yen, T.J. (1998) Mol. Biol. Cell. pp. 2361-2374) recognizes a band with a slightly faster mobility than *Xenopus* ATM.

Figure 6: X-ATM protein following DNA damage.

The level of ATM in extracts from control and γ-irradiated embryos between stage 8 and 10.5 was compared. Embryos were γ-irradiated (40 Gy) using a ^{60}Co source at stage 2. Embryos were collected and crushed at the indicated stages, and the soluble extract was analyzed by 6% SDS-PAGE followed by Western blot analysis. ATM in a mitotic extract (CSF) is also shown.

Figure 7: X-ATM complexes

200 μg of egg extract were electrophoresed under native conditions and immunoblotted with X-ATM antibody (first lane). A band of approximately 450-500kD corresponding to the complex containing X-ATM is observed as indicated. The same sample was electrophoresed in the same polyacrylamide gradient gel under reducing and denaturing conditions (second lane) .The immunoreactive band migrates at 370kDa, the molecular weight of monomeric ATM.

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Acknowledgments:

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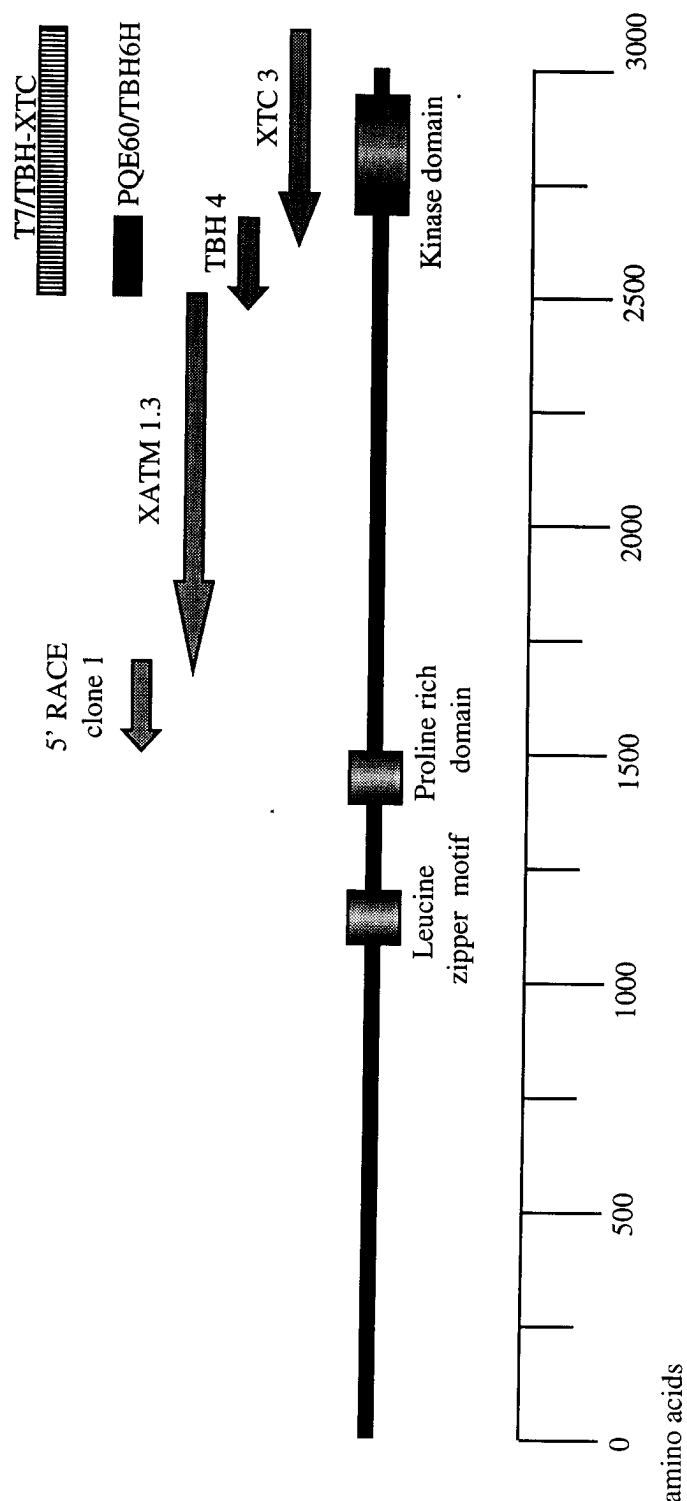Yuille, M.A. & Coignet, L.J. (1998). *Recent Results Cancer Res*, **154**, 156-73.

Figure 1A

Figure 1B

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X-ATM      DVSTRSLLLC LDLLHRVCRA AVTHCEDALE CHHHVIVGSL   40     X-ATM      MFQIKQYNPS QLGVSEWQLE EAQIFWDKEE PSLAREILKQ  840
H-ATM      DVSLRSFSLC CDLLSQVCQT AVTYCKDALE NHLHVIVGTL  1520    H-ATM      IFQIKQYNSV SCGVSEWQLE EAQVFWAKKE QSLALSILKQ  2310
Consensus  DVS.RS..LC .DLL..VC.. AVT.C.DALE .H.HVIVG.L           Consensus  .FQIKQYN.. ..GVSEWQLE EAQ.FW.K.E .SLA..ILKQ

X-ATM      IPLANSQESI QEKVCDLLNF LVIENKDNEN LYHTIKLLDP   80     X-ATM      MINKLEIKSF EVENDSRLRL LYAESLRLCG KWLAETCLES  880
H-ATM      IPLVYEQVEV QKQVLDLLKY LVIDNKDNEN LYITIKLLDP  1560    H-ATM      MIKKLDASC- -AANNPSLKL TYTECLRVCG NWLAETCLEN  2348
Consensus  IPL...Q... Q..V.DLL.. LVI.NKDNEN LY.TIKLLDP           Consensus  MI.KL..... ...N...L.L .Y.E.LR.CG .WLAETCLE.

X-ATM      FPDIPLFKNL RQAHQKIKYS KGPFSLLKEI QNFLSVSVCD  120     X-ATM      PTVIMQNYLE KAVEFAG-YS DGAGEKLQEG RMKAFLSLAR  919
H-ATM      FPDHVVFKDL RITQQKIKYS RGPFSLLEEI NHFLSVSVYD  1600    H-ATM      PAVIMQTYLE KAVEVAGNYD GESSDELRNG KMKAFLSLAR  2388
Consensus  FPD...FK.L R....QKIKYS .GPFSLL.EI ..FLSVSV.D           Consensus  P.VIMQ.YLE KAVE.AG.Y. ......L..G .MKAFLSLAR

X-ATM      SLPLTRLEGL NDLRKQLEQH KDQIKELVRD CQGTPQDSVI  160     X-ATM      FSDAQYQRID NYMKSSEFEN KQALLRKAKE EVGLIKQHKV  959
H-ATM      ALPLTRLEGL KDLRRQLELH KDQMVDIMRA SQDNPQDGIM  1640    H-ATM      FSDTQYQRIE NYMKSSEFEN KQALLKRAKE EVGLLREHKI  2428
Consensus  .LPLTRLEGL .DLR..QLE.H KDQ.....R. .Q..PQD...           Consensus  FSD.QYQRI. NYMKSSEFEN KQALL..AKE EVGL...HK.

X-ATM      ASLVVNLLQL SKNAVHQSNG KEVLEAVGSC LGELGPIDFS  200     X-ATM      QNNRYTVKVE RELQLDECAI LALREDRKRF LCKAVENYIS  999
H-ATM      VKLVVNLLQL SKMAINHTGE KEVLEAVGSC LGEVGPIDFS  1680    H-ATM      QTNRYTVKVQ RELELDELAL RALKEDRKRF LCKAVENYIN  2468
Consensus  ..LVVNLLQL SK.A...... KEVLEAVGSC LGE.GPIDFS           Consensus  Q.NRYTVKV. REL.LDE.A. .AL.EDRKRF LCKAVENYI.

X-ATM      NIALQQHKKD SVYLKADKVF EEKELQCVLV MLTLINNALT  240     X-ATM      CLVSGEEHDM WIFRLCSLWL ENSAVSDVNS MMRQDAQKIP  1039
H-ATM      TIAIQHSKDA S-YTKALKLF EDKELQWTFI MLTYLNNTLV  1719    H-ATM      CLLSGEEHDM WVFRLCSLWL ENSGVSEVNG MMKRDGMKIP  2508
Consensus  .IA.Q..K.. S.Y.KA.K.F E.KELQ.... MLT..NN.L.           Consensus  CL.SGEEHDM W.FRLCSLWL ENS.VS.VN. MM..D..KIP

X-ATM      DHCIQVRSAA ATCLKNILAT KTGAMFWEAC KDKGEPMLLY  280     X-ATM      SHKFLPLMYQ LAARMGTKKM GNPGFHDVLN NLIGRISMDH  1079
H-ATM      EDCVKVRSAA VTCLKNILAT KTGHSFWEIY KMTTDPMLAY  1759    H-ATM      TYKFLPLMYQ LAARMGTKMM GGLGFHEVLN NLISRISMDH  2548
Consensus  ..C..VRSAA .TCLKNILAT KTG..FWE.. K....PML.Y           Consensus  ..KFLPLMYQ LAARMGTK.M G...GFH.VLN NLI.RISMDH

X-ATM      LQPFRAPKKK FLEVEEIQRE GALESLDDPG IWIPQNENHE  320     X-ATM      PHHTLFIILA LANANKDDQL MKAEAVKRSR LTKNAPKQIS  1119
H-ATM      LQPFRTSRKK FLEVPRFDKE NPFEGLDDIN LWIPLSENHD  1799    H-ATM      PHHTLFIILA LANANRDEFL TKPEVARRSR ITKNVPKQSS  2588
Consensus  LQPFR...KK FLEV.....E ...E.LDD.. .WIP..ENH.           Consensus  PHHTLFIILA LANAN.D..L .K.E...RSR .TKN.PKQ.S

X-ATM      SWIKHLTCTL LESGGVKSEV LLLLKPMCEV KADFCQAVVP  360     X-ATM      QLDKERMEAA RHIVDTIKKR RTDMVRDVER LCDAYITLAN  1159
H-ATM      IWIKTLTCAF LDSGGTKCEI LQLLKPMCEV KTDFCQTVLP  1839    H-ATM      QLDEDRTEAA NRIICTIRSR RPQMVRSVEA LCDAYIILAN  2628
Consensus  .WIK.LTC.. L.SGG.K.E. L.LLKPMCEV K.DFCQ.V.P           Consensus  QLD..R.EAA ..I..TI..R R..MVR.VE. LCDAYI.LAN

X-ATM      YIVHNILLND SNQTWRTLLS KNVQRFFTSC CRSLPSSSRS  400     X-ATM      MDANQWKSQR NAIPIPSDQP ITKLNKLHDV VIPTMEIKVD  1199
H-ATM      YLIHDILLQD TNESWRNLLS THVQGFFTSC LRHFSQTSRS  1879    H-ATM      LDATQWKTQR KGINIPADQP ITKLKNLEDV VVPTMEIKVD  2668
Consensus  Y..H.ILL.D .N..WR.LLS ..VQ.FFTSC .R.....SRS           Consensus  .DA.QWK.QR ..I.IP.DQP ITKL..L.DV V.PTMEIKVD

X-ATM      ATPASSDSES EGVARGAVDI ASRRTMLTMV EHLRKQRRPV  440     X-ATM      PSGEYENLVT IVSFKPEFRL AGGLNLPKII DCVGSDGKER  1239
H-ATM      TTPANLDSES EHFFRCCLDK KSQRTMLAVV DYMRRQKRPS  1919    H-ATM      HTGEYGNLVT IQSFKAEFRL AGGVNLPKII DCVGSDGKER  2708
Consensus  .TPA..DSES E..R...D. .S.RTML..V ...R.Q.RP.           Consensus  ..GEY.NLVT I.SFK.EFRL AGG.NLPKII DCVGSDGKER

X-ATM      SGTAFDDNFW LDLNYLEVAM AVQSCAAHFT ALLYSEIYTD  480     X-ATM      RQLVKGQDDL RQDAVMQQVF QMCNTLLQRN SETRKRKLTI  1279
H-ATM      SGTIFNDAFW LDLNYLEVAK VAQSCAAHFT ALLYAEIYAD  1959    H-ATM      RQLVKGRDDL RQDAVMQQVF QMCNTLLQRN TETRKRKLTI  2748
Consensus  SGT.F.D.FW LDLNYLEVA. ..QSCAAHFT ALLY.EIY.D           Consensus  RQLVKG.DDL RQDAVMQQVF QMCNTLLQRN .ETRKRKLTI

X-ATM      KVKQDGEQRT SANRSNARKC LKFEEGSQTL DITGLSEKSK  520     X-ATM      RRYKVVPLSH RSGVLEWCTG TVPIGEYLVN DKDGAHKRYR  1319
H-ATM      KKSMDDQE-- -------KRS LAFEEGSQST TISSLSEKSK  1990    H-ATM      CTYKVVPLSQ RSGVLEWCTG TVPIGEFLVN NEDGAHKRYR  2788
Consensus  K...D..... .......... L.FEEGSQ.. .I..LSEKSK           Consensus  ..YKVVPLS. RSGVLEWCTG TVPIGE.LVN ..DGAHKRYR

X-ATM      EETGISLQDL LMDIYRSIGE PDSLYGCGGG KMLHPLARIR  560     X-ATM      PGDYGSLQCQ RKMMEVQRGR FEEKYQMFLN VCENFRPVFR  1359
H-ATM      EETGISLQDL LLEIYRSIGE PDSLYGCGGG KMLQPITRLR  2030    H-ATM      PNDFSAFQCQ KKMMEVQKKS FEEKYEVFMD VCQNFQPVFR  2828
Consensus  EETGISLQDL L..IYRSIGE PDSLYGCGGG KML.P..R.R           Consensus  P.D....QCQ .KMMEVQ... FEEKY.F.. VC.NF.PVFR

X-ATM      TYEHEAKWGK ALVTFDLEMN LPPVTRQAGI MQALQNFGLC  600     X-ATM      YFCMEKFLDP AMWFEKRLAY TRSVATSSIV GYIVGLGDRH  1399
H-ATM      TYEHEAMWGK ALVTYDLETA IPSSTRQAGI IQALQNLGLC  2070    H-ATM      YFCMEKFLDP AIWFEKRLAY TRSVATSSIV GYILGLGDRH  2868
Consensus  TYEHEA.WGK ALVT.DLE.. .P..TRQAGI .QALQN.GLC           Consensus  YFCMEKFLDP A.WFEKRLAY TRSVATSSIV GYI.GLGDRH

X-ATM      HMLSTYLRGL EHENAEWSSE LQEIHFQAAW RNMQWDDSLP  640     X-ATM      VQNILIDEES AELVHIDLGV AFEQGKILPT PETVPFRLTR  1439
H-ATM      HILSVYLKGL DYENKDWCPE LEELHYQAAW RNMQWDHCTS  2110    H-ATM      VQNILINEQS AELVHIDLGV AFEQGKILPT PETVPFRLTR  2908
Consensus  H.LS.YL.GL ..EN..W..E L.E.H.QAAW RNMQWD....           Consensus  VQNILI.E.S AELVHIDLGV AFEQGKILPT PETVPFRLTR

X-ATM      TKNETSGPGY HESLYRAVQS LRDKEFCGFH DHIKYARVKE  680     X-ATM      DIVDGMGITG VEGVFRRCCE KTMEVMRNNQ EALLTIVEVL  1479
H-ATM      VSKEVEGTSY HESLYNALQS LRDREFSTFY ESLKYARVKE  2150    H-ATM      DIVDGMGITG VEGVFRRCCE KTMEVMRNSQ ETLLTIVEVL  2948
Consensus  ...E..G..Y HESLY.A.QS LRD.EF..F. ...KYARVKE           Consensus  DIVDGMGITG VEGVFRRCCE KTMEVMRN.Q E.LLTIVEVL

X-ATM      VEELCSGSLE SVYSLYPALC RLQAIGELAS VGQMFSRSIT  720     X-ATM      LYDPLFDWTM NPLKALYLQQ ---DEVDLNA TLGGDDPECN  1516
H-ATM      VEEMCKRSLE SVYSLYPTLS RLQAIGELES IGELFSRSVT  2190    H-ATM      LYDPLFDWTM NPLKALYLQQ RPEDETELHP TLNADDQECK  2988
Consensus  VEE.C..SLE SVYSLYP.L. RLQAIGEL.S .G..FSRS.T           Consensus  LYDPLFDWTM NPLKALYLQQ ...DE..L.. TL..DD.EC.

X-ATM      DDGLKDTFLK WQRQSQLLKD SDFEFLEPVL SLRSVILETL  760     X-ATM      RNSCD-SQSV NKVAERVLLR LQEKLTGVEE GMVLSVGGQV  1555
H-ATM      HRQLSEVYIK WQKHSQLLKD SDFSFQEPIM ALRTVILEIL  2230    H-ATM      RNLSDIDQSF DKVAERVLMR LQEKLKGVEE GTVLSVGGQV  3028
Consensus  ...L.....K WQ..SQLLKD SDF.F.EP.. .LR.VILE.L           Consensus  RN.D..QS. .KVAERVL.R LQEKL.GVEE G.VLSVGGQV

X-ATM      LQEEKKQPSQ ESLKDILTKH LLDLSRIARS AGNTQLPEKA  800     X-ATM      NHLIQQAMDP KNLSRLFPGW KAWVZ      1580
H-ATM      MEKEMDNSQR ECIKDILTKH LVELSILART FKNTQLPERA  2270    H-ATM      NLLIQQAIDP KNLSRLFPGW KAWV-       3052
Consensus  ...E...... E..KDILTKH L..LS..AR. ..NTQLPE.A           Consensus  N.LIQQA.DP KNLSRLFPGW KAWV.
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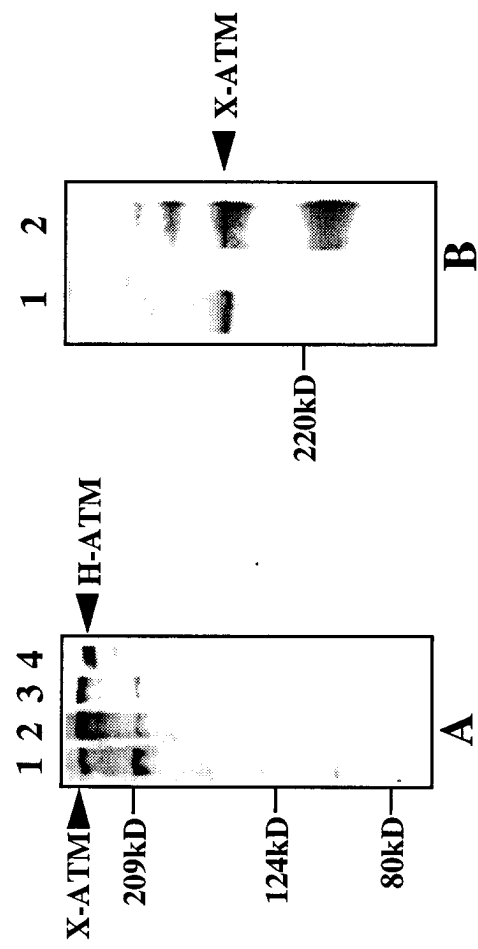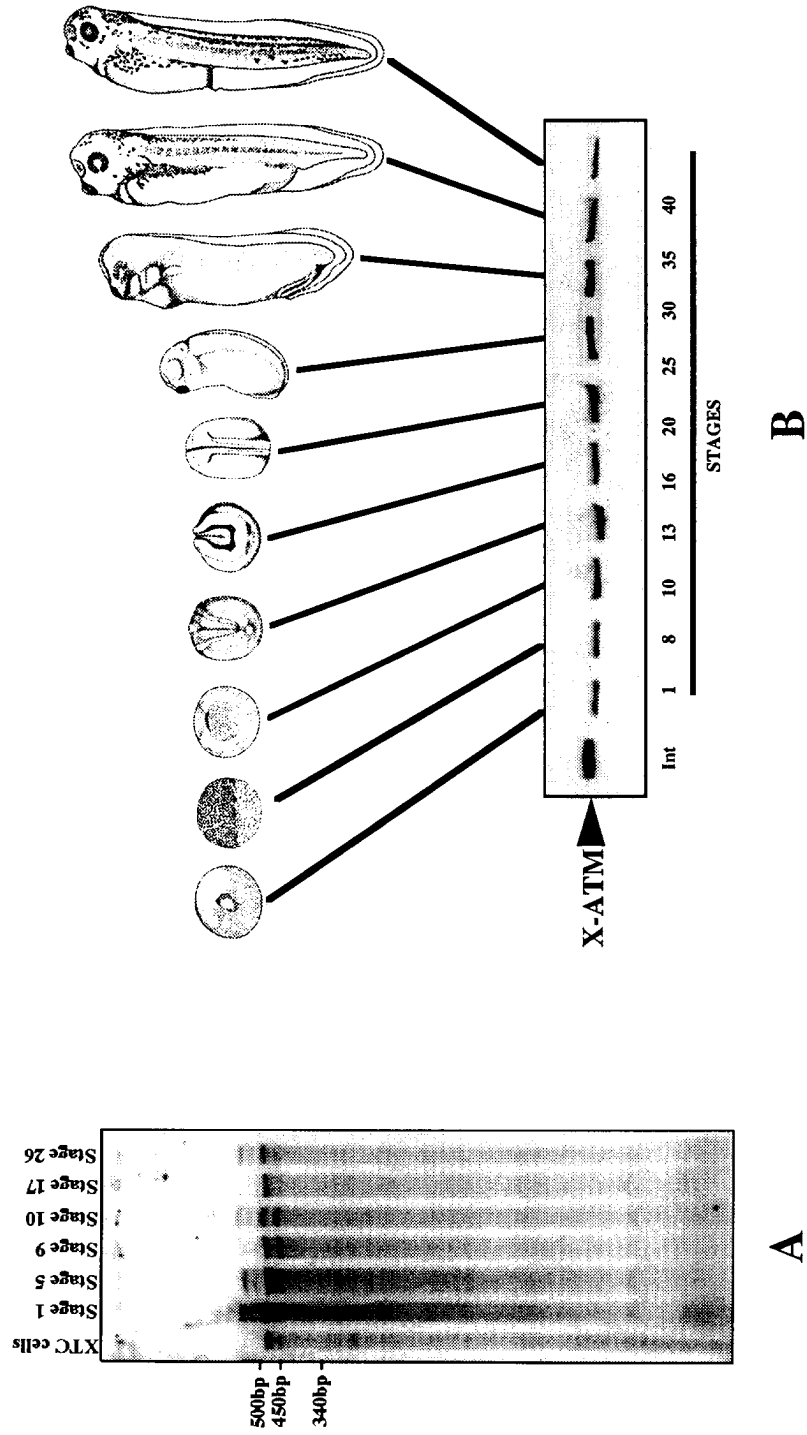
Figure 2

Figure 3

Figure 4

A

Oogenesis

CSF St2 St4 St6

B

Stage 6 Oocytes   Sperm

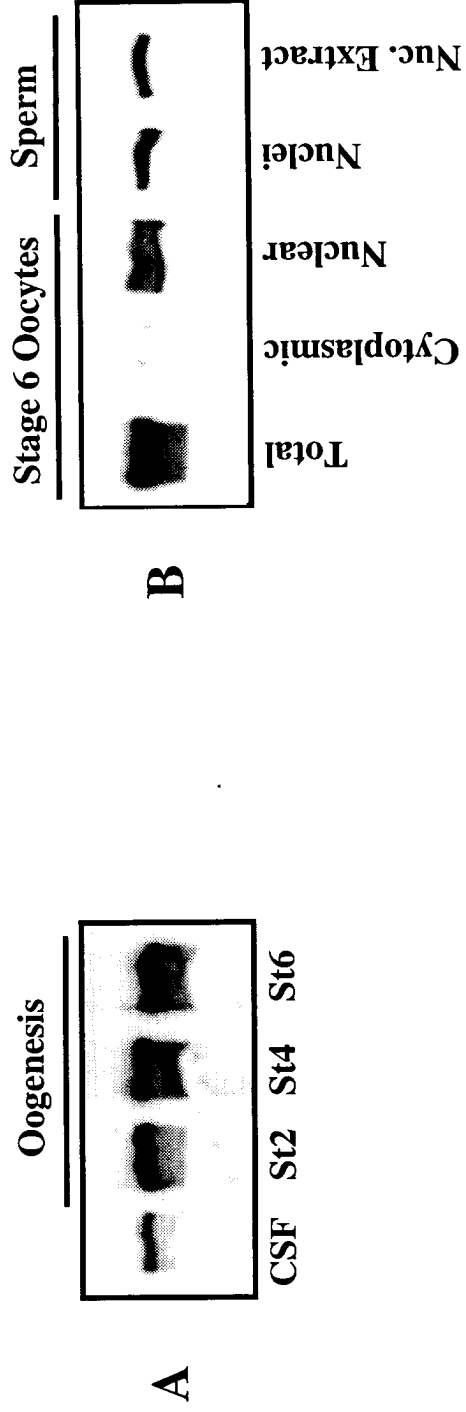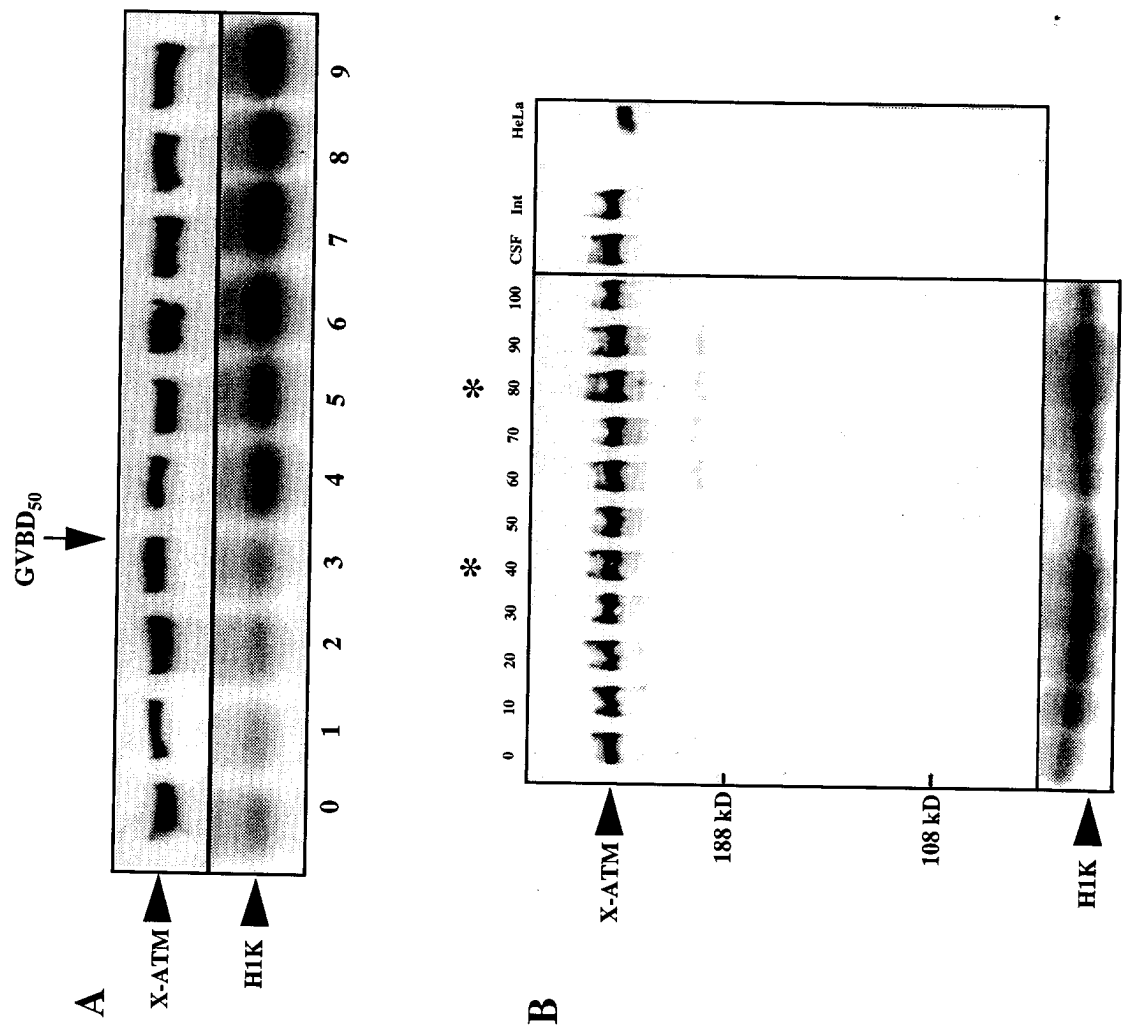Total Cytoplasmic Nuclear Nuclei Nuc. Extract
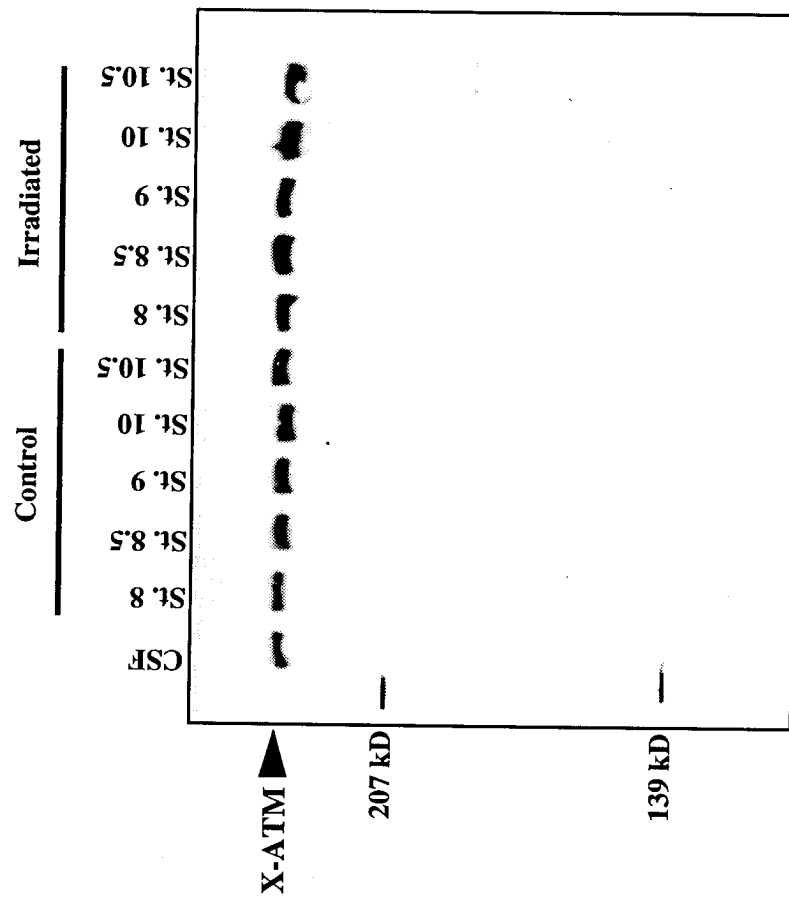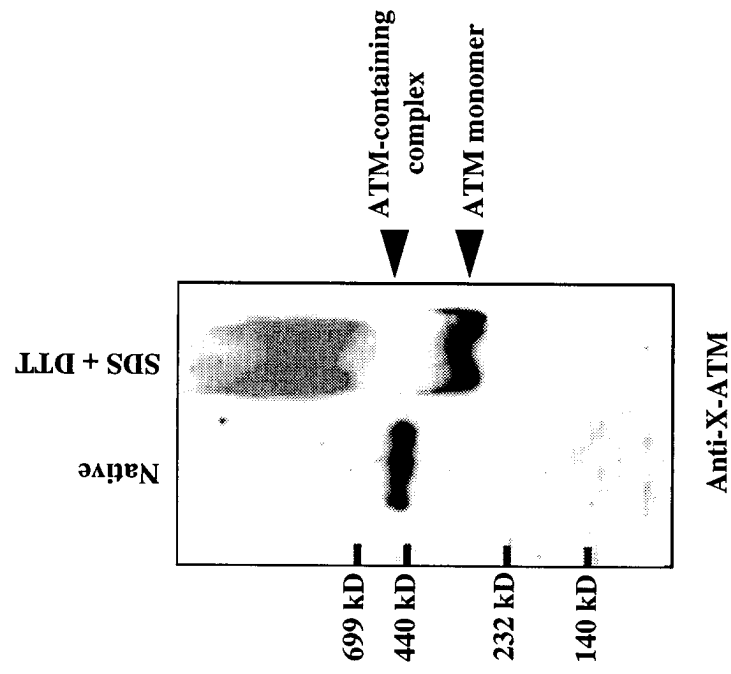
Figure 5

Figure 6

Figure 7

IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A XENOPUS HOMOLOGUE OF ATM

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We used a PCR-based strategy with degenerate oligonucleotide primers to specifically amplify a cDNA fragment of a Xenopus homologue of human ATM. This fragment was further used to isolate cDNA clones from several Xenopus libraries. Libraries screenings in combination with 5' RACE have allowed us to isolate approximately 60% of the predicted full-length Xenopus ATM cDNA. Sequence analysis and homology searches confirmed that the clone was most closely related to human ATM. Xenopus ATM protein (X-ATM) is 85% identical with human ATM within the kinase domain and 66% identical over the rest of the molecule.

We have raised antibodies against a 20 kD polypeptide directly upstream of the kinase domain. These antibodies are very specific for the ATM protein and recognize a single polypeptide of about 350 kD in oocytes, embryos, egg extracts and cell lines from Xenopus. We used affinity purified anti-X-ATM antibodies to characterize the protein expression pattern of X-ATM. We found that X-ATM is expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes. The level of X-ATM protein does not change throughout the synchronous early embryonic cell cycles. In addition, we do not observe any change in the level or mobility of X-ATM protein following gamma-irradiation of the embryos. Finally, we show that X-ATM is present at relatively constant levels during Xenopus development.

Interestingly, we demonstrate that X-ATM is present in a complex with yet unidentified components. We can resolve a high molecular weight complex of approximately 480 kD containing the X-ATM protein. Moreover, we show by manual dissection of oocytes that X-ATM is entirely nuclear in the female germline. In addition, we show that X-ATM protein is associated with sperm chromatin and remains associated with chromatin throughout the cell cycle.